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Rac1 GTPase is a critical factor in phagocytosis in the large yellow croaker *Larimichthys crocea* by interacting with tropomyosin

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A B S T R A C T

The Rho family GTPase Rac1 acts as a molecular switch for signal transduction to regulate various cellular functions. Here, a Rac1 homolog (LcRac1) was identified in large yellow croaker (*Larimichthys crocea),* one of the most economically important marine fishes. The LcRac1 protein was expressed in *Escherichia coli* and purified. Subsequently the specific antibody was raised using the purified fusion protein (GST-LcRac1). LcRac1 was ubiquitously expressed in all 12 tissues we examined, with the highest expression in heart and blood and the weakest expression in head-kidney and spleen. Moreover, time course analysis revealed that LcRac1 expression was obviously up-regulated in liver, spleen and head-kidney after immunization with Poly I:C, LPS and *Vibrio parahemolyticus*. On the other hand, on the basis of protein interaction, it was found that the LcRac1 interacted with Tropomyosin, a crucial protein in the process of phagocytosis. Furthermore, RNAi assays indicated that the phagocytic percentage and phagocytic index were significantly decreased when the LcRac1 gene was silenced by sequence-specific siRNA. Fluorescence microscopy assays revealed FITC-labeled *V. parahemolyticus* were remarkably decreased after LcRac1 was silenced by sequence-specific siRNA at 24 h. These findings implicate the vital role of LcRac1 in innate immunity in the large yellow croaker.

1. Introduction

Rac1 (Ras-related C3 botulinum toxin substrate 1) is a member of the small GTPase Rho GTPase family which also includes Rac2 and Rac3 and Cdc42 [1,2]. Like other Rho GTPases, Rac1 functions as bimolecular switch, cycling between the GDP-bound inactive and GTP-bound active states. Moreover, this cycling is controlled by GTPase activating proteins (GAPs), which enhance their GTPase activity, and guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP to GTP [3]. Rac1 plays vital roles in various molecular signal transduction pathways via regulating the expression of their downstream target genes. It was well documented that Rac1 is the central factor in regulating embryogenesis, cell proliferation, cytoskeleton reorganization, cell-cell adhere junction, growth and cell migration in the past decades [4,5]. Latest research showed that Rac1 homologs are critical in many immune-related processes, such as phagocytosis [6], regulating inflammation-related signal pathways and host immune responses to extracellular stimuli [7]. Rac1 participates in the host immune response against bacterial infection by being involved in almost all aspects of phagocytosis, such as activating NADPH oxidase from which reactive oxygen species (ROS) are generated for bacterial resistance [8,9]. In mammalian cells, Rac1 has also been reported to play a pivotal role in activation of NF-κB pathway via stimulation of cytokines, including IL-1 and TNF-a, while manipulating a parallel pathway involved in the NF-κB signaling pathway [10,11].

Phagocytosis is an important component of immune defence, because of its ability to eliminate microorganisms rapidly and effectively. Phagocytosis is the cellular process of internalizing foreign particles, including bacteria, viruses and fungi [12]. Phagocytosis is well studied in mammalian macrophages. According to previous reports, phagocytosis is comprised of recognition of foreign particles, binding of the foreign particles to the phagocytes, engulfment of cells, and lysis in

Abbreviations: LcRac1, *Larimichthys crocea* Ras-related C3 botulinum toxin substrate 1; ORF, open reading frame; UTR, untranslated region; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; cDNA, DNA complementary to RNA; GST, glutathione S-transferase; IPTG, isopropyl-β-thiogalactopyranoside; GST, reduced glutathione; RNAi, RNA interference

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Table 1
Primers used for LcRac1 and tropomyosin cloning and expression analysis.

Primers	Sequences (5′-3′)	Annealing temperature (°C)	purpose
Rac1-cF1	AGCCGCCGTTAGCTCGC	55	cDNA cloning
Rac1-cR1	AAATCTCAAGTTCTTCC	55	cDNA cloning
β-actin-F	TTATGAAGGCTATGCCCTGCC	60	mRNA expression
β-actin-R	TGAAGGAGTAGCCACGCTCTGT	60	mRNA expression
Rac1-QF	CAGCTACACTACCAATGC	60	mRNA expression
Rac1-QR	CAGGCCCAGGTTCACTGG	60	mRNA expression
Rac1-F1	CGC*GGATCC*ATGCAGGCCATTAAGTG	55	Prokaryotic expression
Rac1-R1	CCG*CTCGAG*TTACAGTATTCTGCACT	55	Prokaryotic expression
TPM-F1	CG*GAATTC*ATGGAGGCCATCAAGAAG	55	Prokaryotic expression
TPM-R1	CCG*CTCGAG*CTACAGAGTAGTCATGTC	55	Prokaryotic expression

phagosomes [13]. Recently, some small GTPases from marine organisms like Ran GTPase [14], Rho GTPase [15] and Rab GTPase [16,17] family are found to be relevant to phagocytosis, implicating that the small GTPases may be involved in innate immunity, contributing to antiviral and antibacterial activity. Moreover, Ran [18], Rab5A [19] and RacGTPase [20] were up-regulated after viral mimic poly I:C, bacteria *Vibrio parahemolyticus* or bacterial lipopolysaccharides (LPS) or parasitic ciliate protozoan (*Cryptocaryon irritans*) stimulation, suggesting small GTPases involved in the immune responses of fish.

Compared to intensive studies of Rac1 in mammals, few studies of Rac1 homolog has been reported in marine organisms such as shrimp [21,22], shellfish [23–25] and Cephalochordata [26] so far. Large yellow croaker (*Larimichthys crocea*) (Richardson 1846), is a commercially important marine fish species in China. In recent years, with the rapid expansion of aquaculture industry of the large yellow croaker, several infectious diseases caused by viruses and bacteria are becoming more and more severe, resulting in large economic loss [27]. Therefore, there is an urgent need to understand the immune system of large yellow croaker to control the diseases and for long-term sustainability.

In this study, the cDNA and genomic DNA encoding Rac1 (LcRac1) were cloned and characterized. The recombinant fusion protein GST-LcRac1 produced by *Escherichia coli* was used to prepare the polyclonal antibody specific against LcRac1 for later on immunological assay. The expression pattern of LcRac1 was determined in healthy state, and the transcription profiles were also surveyed when the large yellow croaker was infected with viral mimic polyinosinic polycytidynic acid (poly I:C), bacteria *V. parahemolyticus* or bacterial lipopolysaccharides (LPS). On the basis of protein interaction, it was found that the LcRac1 interacted with tropomyosin, a crucial protein in the process of phagocytosis. RNAi assays indicated that the phagocytosis activity was significantly decreased when LcRac1 gene was silenced. These data suggested that LcRac1, interacted with tropomyosin, was involved in the phagocytosis response of marine fish for the first time.

2. Materials and methods

2.1. Fish collection and immune challenge

Healthy juvenile large yellow croaker (18.6 ± 1.5 cm in length, 100.7 ± 22.2 g in weight) were collected from a mariculture farm in Ningde, Fujian, China. The fish were acclimatized 10 days at salinity 25–26 psu and temperature 23–26 °C, and fed with a commercial feed. Tissue samples of head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart were sampled from five fish, and frozen in liquid nitrogen, stored at −80 °C until RNA extraction.

For challenge experiments, the fish were cultured at least two weeks and then injected intraperitoneally with 0.5 ml PBS (control group), 0.5 ml poly I:C (27472901, GE Healthcare, England; experimental group 1) at a titer of 0.8 mg/ml, 0.5 ml formalin-inactive Gram-negative bacterium *V. parahaemolyticus* (isolated from diseased fish; experimental group 2) at a titer of 108 cfu/ml, and 0.5 ml LPS (L2880, Sigma, USA; experimental group 3) at a titer of 0.5 mg/ml, respectively. Finally, head-kidney, spleen, and liver of six fish from each above-mentioned group were harvested at 0, 3, 6, 12, 24, 48 and 72 h after injection were sampled and frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction. Each group was set up in triplicate.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA was incubated with RNase-free DNase I (Promega, USA) to remove any genomic DNA contamination. The First strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, USA), following the manufacturer's protocol with Oligo (dT) primer.

2.3. Cloning and sequence analysis of LcRac1

The full length cDNA sequence of LcRac1 was obtained from previous transcriptomic sequences of various tissues from large yellow croaker in our lab (unpublished data). Two specific primers (Rac1-cF1 and Rac1-cR1; Table 1) for RT-PCR were designed based on the cDNA sequence of LcRac1. PCR was performed under the condition of denaturation at 94 °C for 3min, 30 cycles of 94 °C for 30 s, 55 °C annealing for 30s and 72 °C for 1min, followed by a 7min extension at 72 °C. PCR products were cloned into pMD-18T vector (TaKaRa, Dalian, China) and sequenced with Sanger sequencing.

2.4. Amino acids analysis, multiple sequence alignment and phylogenetic tree construction

Sequence homology analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/) and the protein domain features were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple alignments of Rac1 amino acid sequences of large yellow croaker with other species (Table 2) were performed using Clustal-W (http://www.ebi.ac.uk/clustalw/) and a phylogenetic tree of Rac1 protein was constructed through MEGA6 (http://www.megasoftware.net). The protein motifs were analyzed with Expasy Search program (http://au.expasy.org/tools/). The sequences of Rac1 from different species were compared by the NCBI BLASTP search program.

2.5. Expression and purification of recombinant LcRac1/tropomyosin produced by Escherichia coli

The LcRac1 and tropomyosin gene were expressed in *Escherichia coli* BL21 (DE3) as fusion proteins with glutathione S-transferase (GST),

Table 2
Amino acid sequence identities of Rac1 of various organisms.

Species	Common name	Identity (%)	Accession No.
Larimichthys crocea	Large yellow croaker	100	XM_010729684.
Homo sapiens	Human	96	NP_008839.2
Salmo salar	Atlantic Salmon	96	XP_014064538.1
Danio rerio	Zebrafish	95	NP_956065.1
Apis mellifera	Bee	89	XP_623951.1
Gallus gallus	Chicken	96	NP_990348.1
Xenopus laevis	African clawed frog	96	NP_001089332.1
Ctenopharyngodon idella	Grass carp	97	AKS48937.1
Oryzias latipes	Medaka	97	XP_004080205.1
Ceratitis capitata	Fruit fly	91	XP_004519936.1
Sus scrofa	Pig	96	NP_001230514.1
Aedes albopictus	mosquito	91	XP_019539051.1
Helicoverpa armigera	Cotton bollworm	90	XP_021184199.1
Octopus bimaculoides	Octopus	89	XP_014773233.1

respectively. LcRac1 was amplified using the synthesized forward primer Rac1-F1 (Table 1) with a *Bam*HI site (italic), and the reverse primer Rac1-R1 (Table 1) with a *Xho*I site (italic). And TPM-F1 with *Eco*RI site (italic) and TPM-R1 with a *Xho*I site (italic) (Table 1) were used for tropomyosin amplification. The target PCR fragment was inserted into the expression vector pGEX-4T-2 (GE Healthcare Life Science) with downstream of GST after digested with *Bam*HI and *Xho*I enzymes (TaKaRa, Dalian, China). The recombinant plasmid (pGEX-4T-2-LcRac1), (pGEX-4T-2-Tropomyosin) and the vector pGEX-4T-2 as negative control were both transformed into *E. coli* BL21 (DE3). The positive clones of LcRac1 and tropomyosin were screened by PCR with primers Rac1-F1/Rac1-R1 and TPM-F1/TPM-R1 and confirmed by Sanger sequencing, respectively. After incubation at 37 °C overnight, the bacteria containing the LcRac1, Tropomyosin or the control (vector only) were inoculated into fresh LB media at a ratio 1:100. When the OD600 reached 0.6–0.8, the isopropyl-β-thiogalactopyranoside (IPTG) was added to the bacteria at a final concentration of 0.1 mM and incubated for an additional 6 h at 37 °C. The induced and non-induced bacteria were analyzed by SDS-PAGE.

Proteins were purified by the method described previously [19]. Briefly, the bacteria containing the recombinant plasmid were incubated and induced in 1000 ml LB media. The induced bacteria were spun down (4000 ×*g*) at 4 °C, suspended in ice-cold PBS and sonicated for 10 min on ice. The sonicates were mixed with glutathione-agarose beads (Sigma, USA) and incubated at 4 °C for 2 h. The beads were washed with ice-cold PBS, followed by incubation in reducing buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at room temperature for 10 min. After centrifugation at 1000 ×*g* for 5 min, the supernatants were collected and analyzed by SDS-PAGE. The purified protein was stored at −80 °C until use.

2.6. Preparation of antibody and purification

The LcRac1 and the tropomyosin proteins expressed in *E. coli* were used to produce Rac1 or tropomyosin antibody in mice. Firstly the purified GST-LcRac1 or GST-Tropomyosin fusion proteins were used as antigen to immunize mice intradermal injection once every 2 weeks over 8-week period [19]. In more detail, 5 μg antigens were mixed with an equal volume of Freund's complete adjuvant for the first injection. And subsequent injections were conducted using 5 μg of antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma). The immunoglobulin (IgG) fraction was then purified through protein A-Sepharose (Bio-Rad) and stored at −80 °C.

2.7. Real-time PCR analysis of LcRac1 mRNA expression in vivo

The expression of LcRac1 mRNA in heathy fish were assayed in different tissues included head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart. The temporal expression in the liver, spleen and head-kidney challenged with LPS, poly I:C and *V. parahaemolyticus* were assessed using qRT-PCR in an ABI 7500 Real-time Detection System (Applied Biosystems, USA). The housekeeping gene β-actin was used as an internal control for cDNA normalization. The primers of β-actin-F and β-actin-R (Table 1) for β-actin gene were used to amplify a 107-bp fragment. And a 103-bp fragment of LcRac1 cDNA was amplified using the gene-specific primers Rac1-QF/Rac1-QR (Table 1). The PCR product was sequenced to verify the specificity of RT-PCR. The amplification was performed in a total volume of 20 μl, containing 10 μl of 2 × SYBR Green I real-time PCR Master Mix (Tiangen, China), 1 μl of the diluted cDNA, 0.5 μl of each primer (10 μM), and 8 μl of nuclease-free water. The real-time PCR program was 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1min. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only the special PCR product was amplified and detected. The RT-PCR results were analyzed with ABI 7500 SDS software, and the baseline was set automatically by the software. The comparative Ct method ($2^{-\triangle\triangle Ct}$) was used to analyze the relative expression level of Rac1 [19]. SPSS software (version 16.0) was used for the significance test between the test and control group. Differences were considered to be significant at $p < 0.05$ and extremely significant at $p < 0.01$.

2.8. Western blot analysis of LcRac1 protein expression in vivo

Western blot was following the method described previously [19]. Briefly, the protein samples from different tissues including head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart of large yellow croaker were analyzed in a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, USA). The membrane was immersed in blocking buffer at 4 °C overnight, followed by incubation with anti-GST-LcRac1 antibody for 2 h. Then the membrane was incubated in AP-conjugated goat anti-mouse IgG (Sigma, USA) for 1 h and detected with NBT and BCIP solutions (BBI, Canada).

2.9. GST pull-down assay and mass spectral analysis

The purified GST and GST-LcRac1 proteins were separately mixed with glutathione beads and incubated for 2 h at 4 °C. And 100 mg of large yellow croaker muscle was homogenized in 1 ml of GST binding buffer (200 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 4 °C, followed by sonication on ice for 40 s. Then 0.5% NP-40 and 1% TritonX-100 were added into the homogenate. After gentle agitation at room temperature for 1 h, the homogenate was centrifuged at 10,000 ×*g* for 15 min (4 °C). Subsequently the supernatant was subjected to GST-LcRac1 or GST coupled glutathione beads and incubated for 4 h at 4 °C. The beads were washed with GST binding buffer for five times and subjected to SDS-PAGE. The bound proteins, separated by 12% SDS-PAGE, were stained with Coomassie blue for mass spectrometry analysis [19].

MALDI-TOF analysis of protein sample was performed. Briefly, protein bands were excised from the SDS-PAGE gel and subjected to in-gel digestion by trypsin. MALDI-TOF spectra of the peptides were obtained with a time-of-flight delayed extraction MALDI MS (Bruker Autoflex). A nitrogen laser (337 nm) was used to irradiate the sample. Spectra were acquired in reflectron mode in the mass range 600–3500 Da. A near point calibration was applied and a mass tolerance of 100 ppm was used. Data mining was performed using Mascot search engine against the GenBank database.

2.10. Far-western blotting assay

Briefly, during the far-western blotting assay, the GST protein and GST-LcRac1 (or GST-Tropomyosin) fusion proteins were first analyzed

in a 12% SDS-PAGE gel and transferred onto a PVDF membrane (Bio-Rad, USA). The membrane was immersed in blocking buffer at 4 °C for 4 h, followed by incubation with GST-Tropomyosin (or GST-LcRac1) protein for 1 h at room temperature. Then the membrane was washed with PBS for 3 times and incubated with anti-Tropomyosin (or anti-LcRac1) polyclonal antibody for 1 h. At the end, the PVDF membrane was detected with NBT and BCIP solutions (BBI, Canada).

2.11. Synthesis of siRNA and RNAi assay in vivo

The siRNAs used in this study consisted of 21-nucleotide double-stranded RNAs, each strand of which contained a 19-nucleotide target sequence and a two-uracil (U) overhang at the 3' end [28]. According to the design rule for RNAi, a fragment of LcRac1 gene (5′-CTGGAGAGT ACATCCCCACTT-3′) was used as the target siRNA. The sequence was rearranged at random and mutated at one nucleotide, respectively, resulting in the corresponding random-siRNA (5′-CATCGCAGTCACTA GTGAGTT-3′) and mutation-siRNA (5′- CTGGAGAGAACATCCCCA CTT-3′). SiRNAs were synthesized in vitro according to the manufacturer's instruction (Takara, Japan). The formation of double-stranded RNAs was monitored by determining the size shift in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) and quantified by spectrophotometry.

Twenty-four μM of LcRac1-siRNA or mutation-siRNA or random-siRNA were separately delivered into the muscle of the large yellow croaker by injection [28]. The injection volume of each siRNA was 0.1 ml per fish, the siRNAs were dissolved in siRNA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl). At a serial of time after siRNA injection, the fish blood was collected from the ventral sinus cavity of each fish with 29-gauge syringe containing 0.5 ml of pre-cooled (4 °C) anticoagulant solution (500 U ml^{-1} heparin sodium in PBS, pH 7.4). Three fish specimens from each treatment, selected at random, were mixed and subjected to real-time PCR and phagocytosis assays. All assays were repeated in three times.

2.12. Fluorescent labeled bacteria and phagocytosis assay

Bacteria *V. parahemolyticus* were heat-killed at 75 °C for 30 min and were labeled with FITC (Sigma, America) for 1 h at 25 °C. Then phagocytosis assay of fish blood was measured [29]. Briefly hemocytes were resuspended in PBS at 10^6 cells ml^{-1}. And 100 ml of hemolymph was incubated with FITC-labeled bacteria at 28 °C for 30 min. The mixture was smeared onto a poly-L-lysine-coated glass slide and allowed to fix for 0.5 h at room temperature. Subsequently, the hemocytes were stained with propidium iodide (PI, Sigma, America) at the final concentration of 20 mg ml^{-1} for 1 min. Then, the non-adherent cells were removed with PBS and the Trypan blue stain was added to quench any free floating and adherent bacteria. After incubation for 20 min, the cells were washed twice with PBS and one drop of anti-fade solution was added before mounting to examine the hemocytic phagocytosis under a fluorescence microscope. Hemocytes (at least 200/slide) were counted at 800× magnification. The phagocytic percentage (PP) and phagocytic index (PI) were calculated as follows: PP = (no. of cells ingesting bacteria / no. of cells observed) × 100% and PI = (total no. of bacteria ingested / no. of cells ingesting bacteria). The assays were conducted in triplicate. The data were analyzed by *t*-test using the Science Analysis Software (www.ats.ucla.edu/software).

3. Results

3.1. Sequence analysis of LcRac1 cDNA

The full-length cDNA sequence of the LcRac1 of large yellow croaker (GenBank Accession No. XM_010729684) was 843 bp in length with an open reading frame (ORF) of 579 bp encoding 192 amino acids (Fig. 1). Sequence analysis revealed that the base sequence surrounding the methionine start codon (CCGATGC) of the LcRac1 gene was consistent with the Kozak rule for efficient eukaryotic translation initiation as PuNNATGPu described by Kozak [30]. The 5′-terminal untranslated region (UTR) and the 3′-terminal UTR was 84 and 167 bp, respectively. A stop codon (TAA) was included and the polyadenylation signal (AATAAA) was found at 77 bp upstream of the poly (A) tail (Fig. 1). The putative molecular weight of LcRac1 was 21.45 kDa and the estimated isoelectric point was 8.34. There was a RHO domain (residues 6–179) in the LcRac1 protein (Fig. 1).

Three-dimensional molecular modeling of LcRac1 was generated using human Rac1 as the template (Fig. 2). The rate of consistency between LcRac1 and the template was 90.7%. An N-terminal lobe (N-lobe) was made up of 6 β-strands and 6 α-helix, and the connecting C-terminal lobe (C-lobe) was almost comprised of α-helices. Mg^{2+} ligand was found between the two lobes. Additionally, an exclusive effector loop for binding homology region 1b (HR1b) domain of protein kinase C-related kinase 1 (PRK1) [31] was found in C-terminal region of LcRac1. Sequence alignment data showed that the deduced amino acid of LcRac1 shared relative highest identities with Medaka (*Oryzias latipes*) Rac1 (97%) and Grass carp (*Ctenopharyngodon idella*) Rac1 (97%). Homology analysis revealed that LcRac1 shared 89%–97% identities with those Rac1 from other eukaryotic organisms (Fig. 3, Table 2).

3.2. Phylogenetic analysis

A phylogenetic tree was constructed using the neighbor-joining (NJ) method to investigate the relationship between LcRac1 and other known Rac1 proteins in other species (Fig. 4). The phylogenetic tree revealed that the known Rac1 proteins can be divided into two classes. Class 1 contained the vertebrate Rac1 proteins *Hs*Rac1 (*Homo sapiens*), *Ss*Rac1 (*Salmo salar*), *Dr*Rac1 (*Danio rerio*), *Gg*Rac1 (*Gallus gallus*), *Xl*Rac1 (*Xenopus laevis*), *Ci*Rac1 (*Ctenopharyngodon idella*), *Ol*Rac1 (*Oryzias latipes*) and SsRac1 (*Sus scrofa*). Class 2 contained the invertebrate Rac1 proteins *Ob*Rac1 (*Octopus bimaculoides*), *Ha*Rac1 (*Helicoverpa armigera*), *Ab*Rac1 (*Aedes albopictus*), *Am*Rac1 (*Apis mellifera*) and *Cc*Rac1 (*Ceratitis capitata*). Among the class 1 members, *Lc*Rac1 was mostly closed related to the fish Rac1 proteins. The Rac1 amino acid identity among large yellow croaker, Medaka and Grass carp were 97% (Table 2). The observed relationships within this cluster reflected the taxonomic positions of the species (Fig. 4), indicating that the LcRac1 protein is a member of Rac1 GTPase family.

3.3. Expression and purification of recombinant protein GST-LcRac1

The LcRac1 ORF was cloned into a pGEX-4T-2 vector to generate a recombinant LcRac1 fused with GST (GST-LcRac1). A transformant of *E. coli* BL21 harbouring this expression plasmid was selected, cultured and induced by IPTG. After induction, the total proteins were extracted and analyzed by SDS-PAGE. Results showed that a band approximately 47 kDa corresponding to the fusion protein GST-LcRac1 was observed (Fig. 5, lane 4), while no protein band located at that position was found in both induced and non-induced control bacteria (Fig. 5, lanes 1 and 2). This evidence indicated that the LcRac1 ORF was expressed in *E. coli* BL21. Furthermore, the recombinant protein GST-LcRac1 was purified using affinity chromatography (Fig. 5, lane 5).

3.4. Expression analysis of LcRac1 in tissues

To understand the expression patterns of LcRac1 in healthy fish, quantitative real-time PCR was used to analyze the mRNA expression level of LcRac1 in multiple tissues including head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart. The transcriptional level of LcRac1 in each tissue was normalized to β-actin. The results showed that LcRac1 had the highest expression in heart, followed by in blood, brain, gill, skin, liver, intestine, stomach,

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1    AGCCGCCGTTAGCTCGCTAAGCTAACCGAGCTCGACCCGCTGTAACGCTGACTGTGGGCT   60
61   GCTAGCGAGAGAGAGAGCCGCCCGATGCAGGCCATTAAGTGTGTGGTAGTGGGGGACGGG  120
1                                 M  Q  A  I  K  C  V  V  V  G  D  G   12
121  GCTGTGGGTAAAACATGCCTGCTCATCAGCTACACTACCAATGCCTTCCCTGGAGAGTAC  180
13   A  V  G  K  T  C  L  L  I  S  Y  T  T  N  A  F  P  G  E  Y   32
181  ATCCCCACAGTATTTGACAACTACTCTGCCAATGTGATGGTTGACGGGAAACCAGTGAAC  240
33   I  P  T  V  F  D  N  Y  S  A  N  V  M  V  D  G  K  P  V  N   52
241  CTGGGCCTGTGGGATACAGCAGGACAGGAGGACTATGACAGGCTCAGGCCTCTGTCCTAC  300
53   L  G  L  W  D  T  A  G  Q  E  D  Y  D  R  L  R  P  L  S  Y   72
301  CCACAGACAGATGTGTTCCTGATTTGCTTTTCACTAGTCAGTCCAGCCTCCTTTGAAAAC  360
73   P  Q  T  D  V  F  L  I  C  F  S  L  V  S  P  A  S  F  E  N   92
361  GTCCGTGCCAAGTGGTACCCTGAGGTGAGACATCATTGCCCCAACACACCCATCATCCTG  420
93   V  R  A  K  W  Y  P  E  V  R  H  H  C  P  N  T  P  I  I  L  112
421  GTGGGCACCAAGCTGGACCTGAGAGACGACAAGGACACTATCGAAAAACTCAAGGAGAAG  480
113  V  G  T  K  L  D  L  R  D  D  K  D  T  I  E  K  L  K  E  K  132
481  AAGCTCTCCCCCATTATTTACCCTCAGGGCTTGGCCATGGCTAAGGAAATAAGCTCAGTG  540
133  K  L  S  P  I  I  Y  P  Q  G  L  A  M  A  K  E  I  S  S  V  152
541  AAGTACCTGGAGTGCTCAGCTTTGACGCAGCGCGGCCTTAAGACAGTGTTCGACGAAGCC  600
153  K  Y  L  E  C  S  A  L  T  Q  R  G  L  K  T  V  F  D  E  A  172
601  ATCAGGGCGGTCCTGTGCCCCCCACCCATCAAGAAGAGGAAGAGGAAGTGCAGAATACTG  660
173  I  R  A  V  L  C  P  P  P  I  K  K  R  K  R  K  C  R  I  L  192
661  TAAAAGAATGAATCCCAGGGAGTAGAAAGTATGATTGCAGTCAGAACTCACTTATTCAGA  720
     *
721  GGGAAGATGATGATGACTGGGTTCCCCAATAAACTATTCACACCCTGTGTCCTTGCTTAC  780
781  ATAGAAGCTCTGGTAGATCAGACTTCCATCTGGAAGAACTTGACAGATTTAAAAAAAAAA  840
841  AAA                                                           843
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Fig. 1. Full-length nucleotide sequence and deduced amino acid sequence of LcRac1 (GenBank Accession No. XM_010729684). The bold letters in box indicated the start codon (ATG), the stop codon (TAA) and the polyadenylation signal sequence (AATAAA). The conserved RHO domain is shaded and the sequence underlined represented nuclear localization signal.

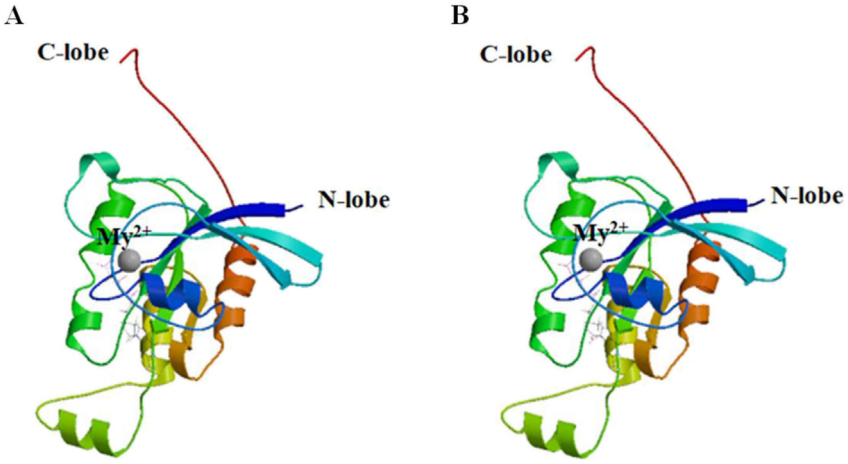

Fig. 2. 3D structure prediction of LcRac1 protein (A) and human Rac1 protein (B). Mg^{2+} is indicated by a grey sphere.

kidney, muscle and spleen, and had the lowest expression level in head-kidney (Fig. 6A).

To characterize the tissue distribution of LcRac1 protein in large yellow croaker, specific antibodies were prepared in mice immunized with the purified recombinant GST-LcRac1 protein. Western blot showed that the antibody against LcRac1 strongly reacted with a specific band corresponding to the Rac1 protein in the 12 large yellow croaker tissues (head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart), indicating that the expression of LcRac1 protein was restricted to the same organs as those of its transcript (Fig. 6B).

3.5. Up-regulation of LcRac1 post poly I:C, LPS and V. parahemolyticus challenge

To further determine the function of LcRac1 in immune response, its temporal expression patterns in head-kidney, spleen and liver under stimulation of LPS, poly I:C and *V. parahemolyticus* infection by qPCR using the β-actin as an internal control were also assayed.

The expression profile of LcRac1 was detected in head-kidney, and the results revealed that LcRac1 was significantly increased at 3 h post injection (hpi) ($P < 0.05$), with gradually recovered to the control group from 6 hpi to 24 hpi, and reached to the peak value at 48 hpi

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Larimichthys crocea      1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Salmo salar              1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Oryzias latipes          1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Danio rerio              1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Ctenopharyngodon idella  1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Xenopus laevis           1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Homo sapiens             1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Sus scrofa               1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Gallus gallus            1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPL 70
Helicoverpa armigera     1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPINLGLWDTAGQEDYDRLRPL 70
Octopus bimaculoides     1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPINLGLWDTAGQEDYDRLRPL 70
Apis mellifera           1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPINLGLWDTAGQEDYDRLRPL 70
Ceratitis capitata       1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDAKPINLGLWDTAGQEDYDRLRPL 70
Aedes albopictus         1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPINLGLWDTAGQEDYDRLRPL 70
Clustal Consensus        1   ********************************************.**:****************** 69

Larimichthys crocea      71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLSPIIYP 140
Salmo salar              71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
Oryzias latipes          71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPVTPIILVGTKLDLRDDKDTIEKLKEKKLSPIIYP 140
Danio rerio              71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
Ctenopharyngodon idella  71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
Xenopus laevis           71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
Homo sapiens             71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
Sus scrofa               71  SYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLRDDKDTIEKLKEKKLTPITYP 140
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Helicoverpa armigera     71  SYPQTDVFLICFSLVNPASFENVRAKWFPEVRHHCPSTPIILVGTKLDLREDKDTIEKLKDKKLAPITYT 140
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Ceratitis capitata       71  SYPQTDVFLICFSLVNPASFENVRAKWYPEVRHHCPNVPIILVGTKLDLRDDKATIEKLKEKKLTPITYP 140
Aedes albopictus         71  SYPQTDVFLICFSLVNPASFENVRAKWYPEVRHHCPNIPIILVGTKLDLRDDKQTVDKLRDKKLSPITYP 140
Clustal Consensus        70  **************:.**********:*** ****  ***********:** *:::*::***:** *. 132

Larimichthys crocea      141 QGLAMAKEISSVKYLECSALTQRGLKTVFDEAIRAVLCPPPIKKRKRKCRIL 192
Salmo salar              141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCSLL 192
Oryzias latipes          141 QGLAMAKEISAVKYLECSALTQRGLKTVFDEAIRAVLCPPPDRKKRKRKCRIL 192
Danio rerio              141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKRRRRRCLLL 192
Ctenopharyngodon idella  141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCKIL 192
Xenopus laevis           141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRRCRLL 192
Homo sapiens             141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL 192
Sus scrofa               141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL 192
Gallus gallus            141 QGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPPPVKKRKRKCLLL 192
Helicoverpa armigera     141 QGLGMSKEISAVKYLECSALTQKGLKTVFDEAIRAVLCPVQPIKPRRKCTLL 192
Octopus bimaculoides     141 QGLAMAKEITAVKYLECSALTQKGLKMVFDEAIRAVLCPKPKPCKRHKCKIF 192
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Ceratitis capitata       141 QGLAMAKEIGAVKYLECSALTQKGLKTVFDEAIRSVLCPVMRVKRSRKCALL 192
Aedes albopictus         141 QGLAMAKEIGAVKYLECSALTQKGLKTVFDEAIRAVLCPIIPVKNKRKCSIL 192
Clustal Consensus        133 ***.*:*** :**********:*** *******:****      ::* :: 173
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Fig. 3. Multiple alignment of LcRac1 protein with other known Rac1 family proteins. GenBank accession numbers of these proteins are listed in Table 2.

(8.3-fold) after poly I:C stimulation. After *V. parahemolyticus* stimulation, sharp up-regulation of LcRac1 was detected at 3 hpi ($P < 0.05$), whereas no significant change has been found after LPS infection (Fig. 7A).

In spleen, the expression profile of LcRac1 was up-regulated at 3 hpi following poly I:C stimulation, and the expression level was also significantly changed ($P < 0.05$) at 6 hpi and 48 hpi with *V. parahemolyticus* and 12 hpi with LPS stimulation (Fig. 7B).

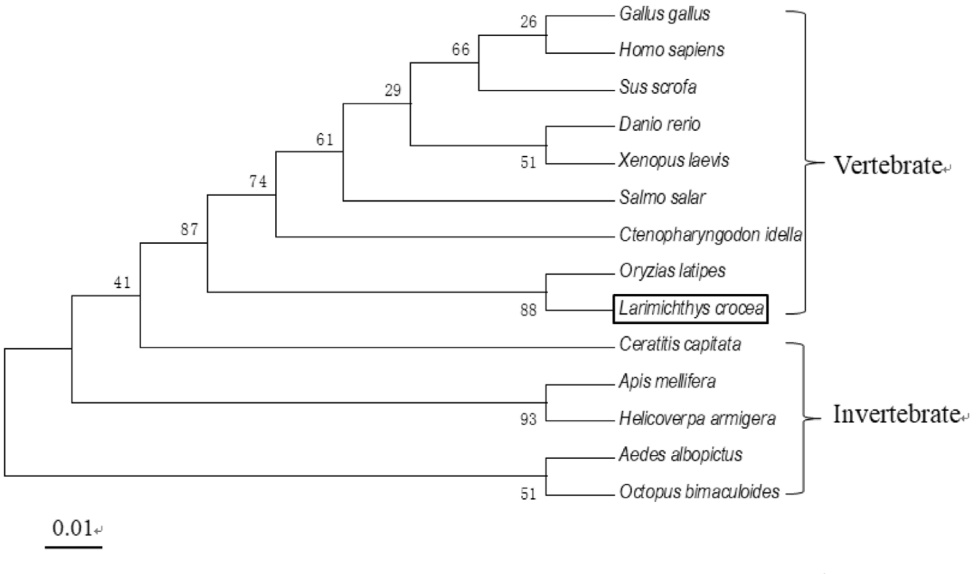

Fig. 4. Phylogenetic relationship of LcRac1 with known Rac1 family proteins. Complete amino acids sequences were aligned using CLUSTALW, and the tree was constructed with NJ method in MEGA6. The number at each node indicated the percentage of bootstrapping after 1000 replications. GenBank accession numbers of these proteins are listed in Table 2. The scale bar represents the number of substitutions per site.

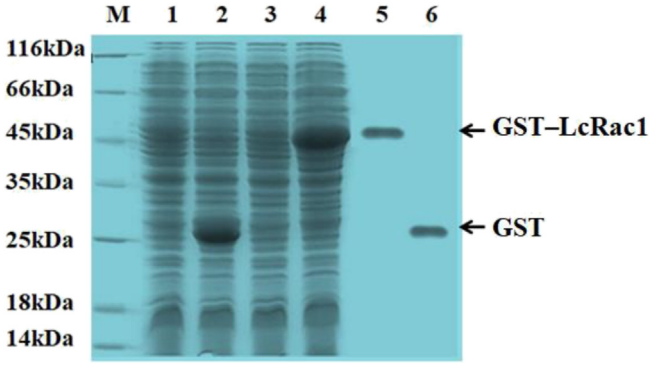

Fig. 5. The production and purification of recombinant LcRac1 in *E. coli* were examined by 12% SDS-PAGE. And the arrows indicated purified LcRac1 and GST proteins. Lane M, molecular weight standard; Lane 1, pGEX-4T-2-BL21 (the vector only as a control), without IPTG induced; lane 2, pGEX-4T-2-BL21, induced; Lane 3, pGEX-4T-2 vector containing LcRac1 gene without IPTG induced; lane 4, pGEX-4T-2 vector containing LcRac1 gene after IPTG induction for 6 h (supernatant); Lane 5, purified GST-LcRac1 fusion protein; and Lane 6, purified GST protein.

In addition, the results showed that the expression level of LcRac1 was significantly up-regulated at 3 hpi of all three stimuli in liver, with 6 times stronger than that of the control group ($P < 0.05$), and that expression level maintained up-regulated at 24 hpi following LPS stimulation (Fig. 7C). *V. parahemolyticus* and LPS stimulation also increased the transcripts of LcRac1 at 3 hpi and 24 hpi ($P < 0.05$), whereas no significant change has been shown from 48 to 72 h after poly I:C injection, but a tiny increase at 48 hpi with LPS stimulation compared to the control group ($P < 0.05$) (Fig. 7C).

3.6. LcRac1 depletion by RNAi led to a defect in hemocytic phagocytosis

In an attempt to further elucidate the role of LcRac1 on phagocytosis against pathogens, RNAi assays were conducted in this study. The real-time PCR results showed that a decrease in the LcRac1 mRNA level of fish blood from 12 h onwards after injection with the LcRac1 gene-specific siRNA (LcRac1-siRNA), and the LcRac1 transcript was reduced dramatically from 24 h to 96 h, and dropped to the lowest at 48 h (Fig. 8A). However, the LcRac1 gene was normally transcribed when treated with random-siRNA (Fig. 8A), suggesting that the LcRac1-siRNA was specific. To investigate whether the LcRac1-siRNA was only targeting the LcRac1 mRNA rather than inducing a non-specific RNAi, a

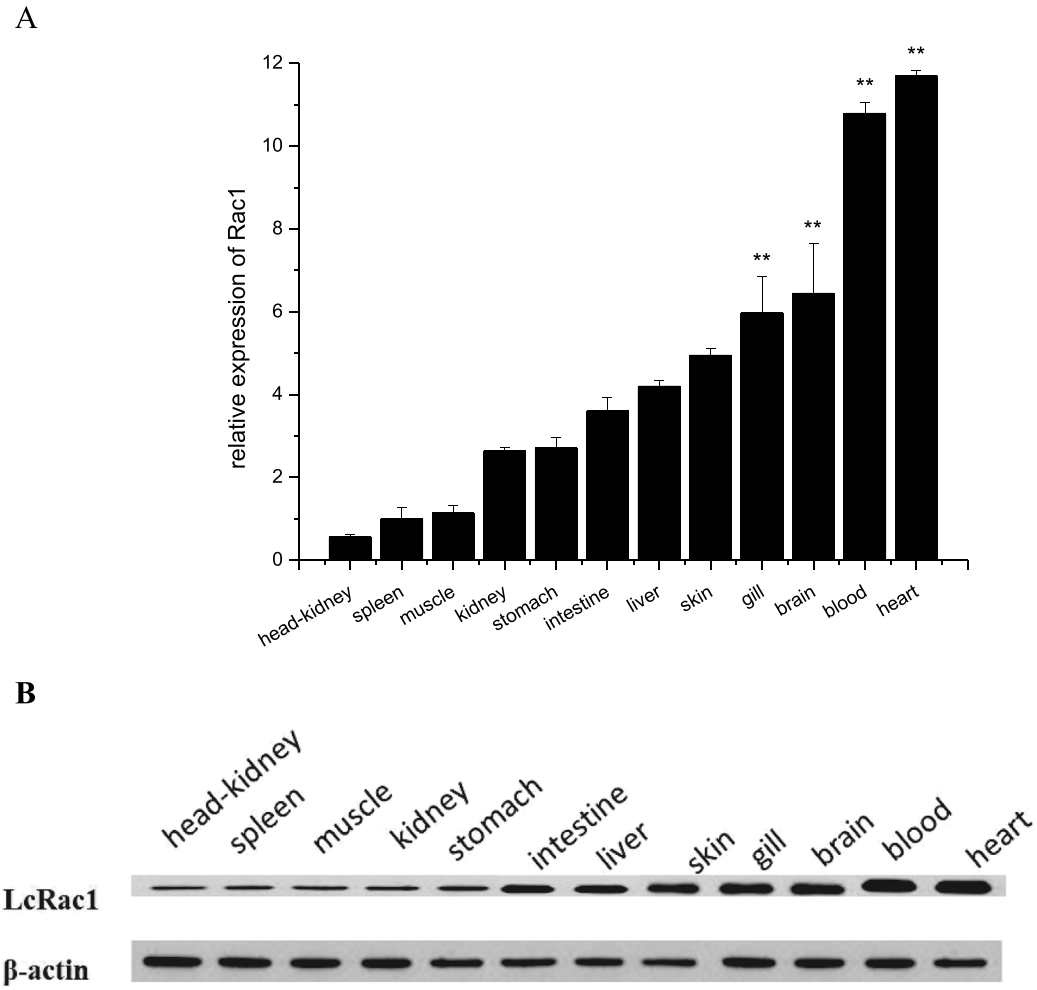

Fig. 6. Tissue distribution of LcRac1 in the large yellow croaker. (A) The expression of LcRac1 mRNA as determined by real-time quantitative PCR. Tissue samples included head-kidney, spleen, muscle, kidney, stomach, intestine, liver, skin, gill, brain, blood and heart were collected from six large yellow croaker. (B) The expression of LcRac1 protein as determined by Western blot analysis using the β-actin protein as a control. The anti-GST-LcRac1 antibody was used. Significant differences are indicated with asterisks. *$P < 0.05$, **$P < 0.01$.

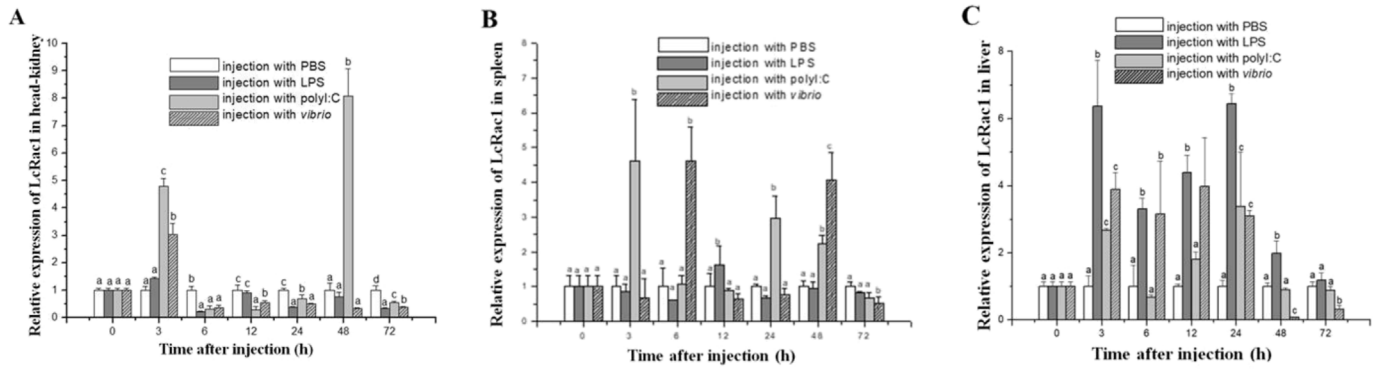

Fig. 7. The temporal expression of LcRac1 in head-kidney (A), spleen (B) and liver (C) challenged by LPS, PolyI:C or *V. parahaemolyticus* stimulation at 0 h-72 h by qRT-PCR. The expression of control group was set as 1.0, and the values were shown as means ± S.E (n = 6). Stages with the same letter indicate no significant difference of LcRac1 gene expression level between the different treatment groups.

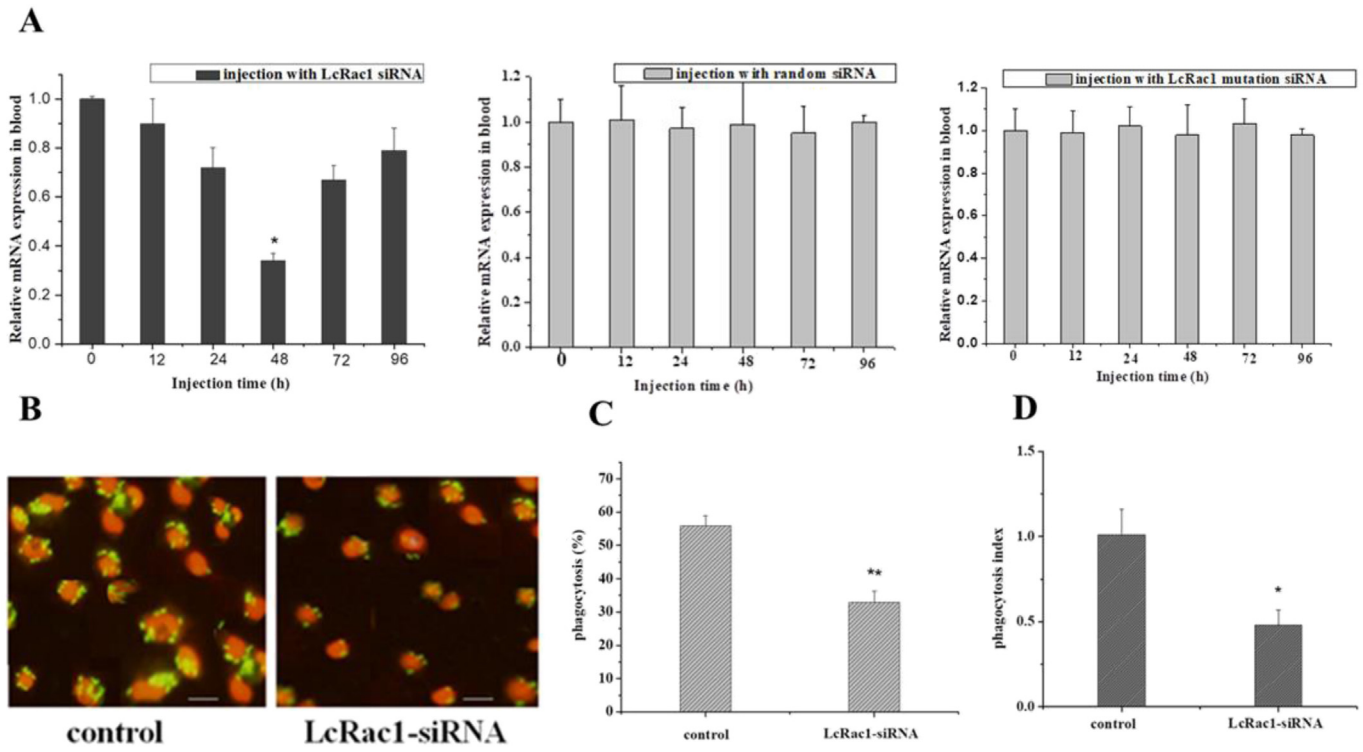

Fig. 8. Effect of the LcRac1 gene silencing on hemocytic phagocytosis by RNAi in the large yellow croaker. (A) Real-time PCR detection of LcRac1 mRNA using the total RNA extracted from blood of large yellow croaker. The headings showed the siRNAs used for injection. β-actin was used as an internal control. Each experiment was performed in triplicate and each column showed the mean of triplicate assays. (B) Hemocytic phagocytosis assays were conducted at 0 h and 48 h after injection of LcRac1-siRNA using FITC-labeled *V. parahemolyticus*. The red was blood cells, and the green was bacteria. The bar is 25 μm. (C) The phagocytic percentages. Each column represents the mean of triplicate assays. Error bars indicated the standard deviations. (D) The phagocytic index. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

single-nucleotide variation in the LcRac1-siRNA (mutation-siRNA) was characterized. The results indicated that the LcRac1 transcript kept relatively consistent (Fig. 8A), showing that the LcRac1-siRNA could silence the expression of the LcRac1 gene, but not other genes. In conclusion, the data displayed that the sequence-specific LcRac1-siRNA efficiently inhibited the transcription and translation of the LcRac1 gene. In the case of the LcRac1 silencing by RNAi at 48 h, the fish hemocytic phagocytosis activity was evaluated using FITC-labeled bacteria (Fig. 8B). And the results showed that for the treatment of injection with LcRac1-siRNA at 48 h, the phagocytic percentage was decreased from 55.8% to 32.9% (Fig. 8C), and the phagocytic index of LcRac1 dropped to 0.48, (Fig. 8D), whereas there was no difference of phagocytosis for the mutation-siRNA and random-siRNA (data not shown). The above results clearly indicated that the LcRac1 protein was crucial in hemocytic phagocytosis in the large yellow croaker.

3.7. LcRac1 protein interacted with tropomyosin in vivo in the large yellow croaker

In an attempt to identify the immune pathway in which the LcRac1 protein was involved, protein-protein interaction was investigated. The GST pull-down assays with the LcRac1 protein were conducted using muscle of large yellow croaker. The results showed that a protein was specifically bound with the LcRac1 protein. Based on mass spectrometric identifications, the interacting protein was found to be tropomyosin (Fig. 9A). To confirm this complex, tropomyosin was cloned and expressed. The full-length cDNA sequence of the tropomyosin of large yellow croaker (GenBank Accession No. XM_010747216) was 1265 bp

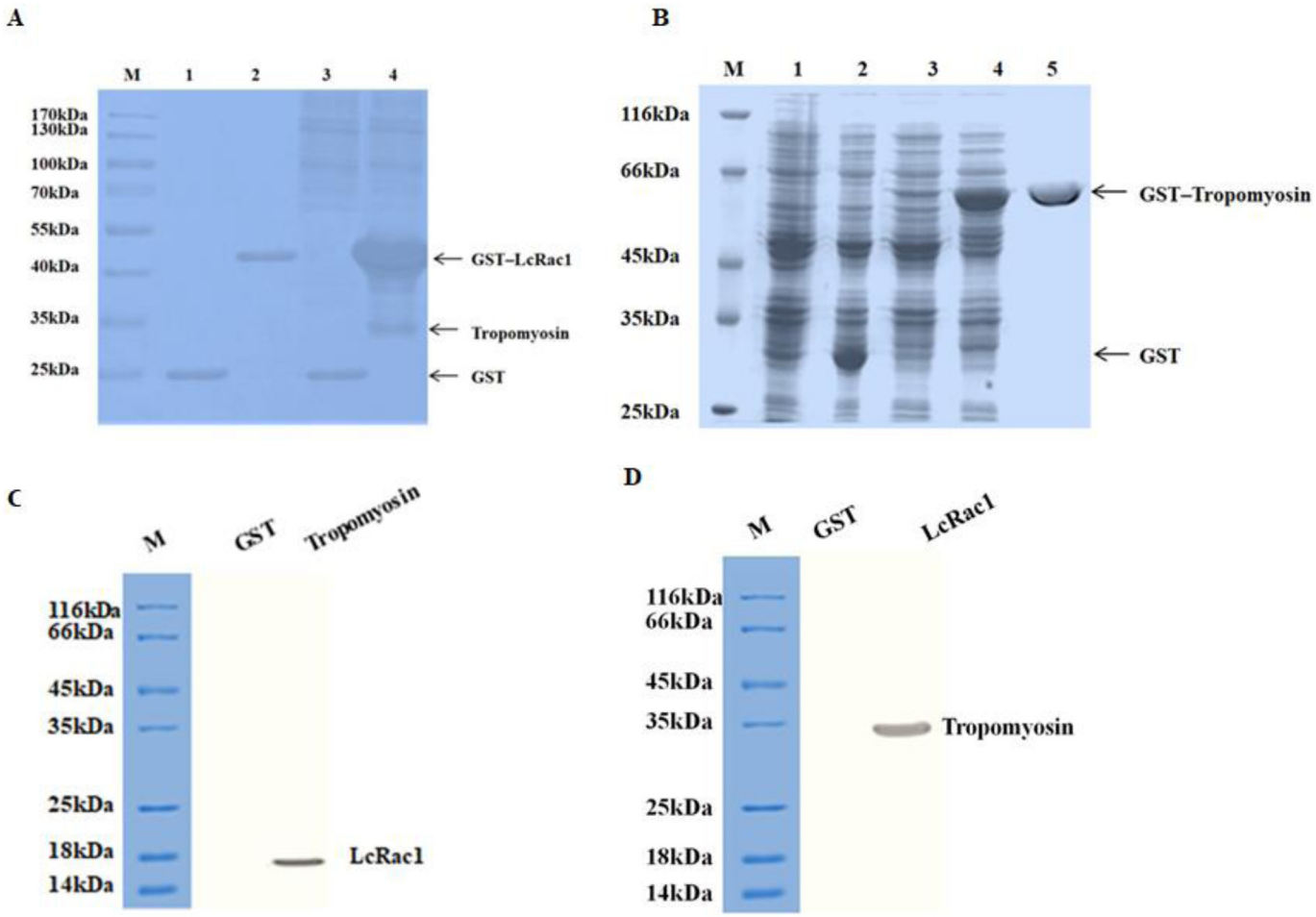

Fig. 9. Interaction between LcRac1 and tropomyosin. (A) GST pull-down assay with LcRac1 protein using large yellow croaker muscle. The eluates were separated via SDS-PAGE stained with coomassie blue, followed by mass spectrometric identification. The arrow indicated proteins identified by mass spectrometry. Lane M, protein molecular mass markers; 1, purified GST protein; 2, purified GST-LcRac1 fusion protein; 3, GST pull-down with GST protein as control; 4, GST pull-down with GST-LcRac1 fusion protein. The arrow indicated that tropomyosin interacted with LcRac1 protein. (B) The production and purification of recombinant tropomyosin in *E. coli* were examined via 12% SDS-PAGE. And the arrows indicated purified tropomyosin and GST proteins. Lane M, molecular weight standard; Lane 1, pGEX-4T-2-BL21 (the vector only as a control), without IPTG induced; lane 2, pGEX-4T-2-BL21, induced; Lane 3, pGEX-4T-2 vector containing tropomyosin gene without IPTG induced; lane 4, pGEX-4T-2 vector containing tropomyosin gene after IPTG induction for 6 h (supernatant); Lane 5, purified GST-Tropomyosin fusion protein. (C) Far-western blotting with specific LcRac1 antibody. Lanes M, protein molecular mass markers; GST, GST protein; Tropomyosin, GST-Tropomyosin fusion protein. (D) Far-western blotting with specific tropomyosin antibody. Lanes M, protein molecular mass markers; GST, GST protein; LcRac1, GST-LcRac1 fusion protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in length with an open reading frame (ORF) of 855 bp encoding 284 amino acids. Tropomyosin was transformed into BL21 to produce recombinant protein GST-Tropomyosin. We observed that a band at approximately 60 kDa corresponding to the fusion protein GST-Tropomyosin when separating the total proteins extracted from the BL21 through SDS-PAGE (Fig. 9B), which was then purified to prepare a polyclonal antibody specifically against tropomyosin. Far-western blotting with specific LcRac1 and tropomyosin antibody also indicated the interaction between LcRac1 and tropomyosin in vitro (Fig. 9C and D), again suggesting that the LcRac1 and the cytoskeleton protein tropomyosin formed a complex.

4. Discussion

In previous study, a Rac GTPase was identified from the large yellow croaker [20]. Herein, we identified a novel Rac1 homolog - LcRac1 from this marine fish. Its full-length cDNA was 843 bp, including an open reading frame (ORF) of 579 bp encoding a polypeptide of 192 amino acids. The LcRac1 protein contained a RHO domain (residues 6–179), which was a characteristic sequence of the Rac1 protein [32]. And one cysteine residue at the C terminus can be isoprenylated, which enables Rac1 proteins to associate and target to cell membranes by a crucial post-translational modification [33]. In our phylogenetic analysis, LcRac1 clustered with Rac1 sequences of other fish species (Fig. 3). Meanwhile, pairwise multiple alignment presented the amino acid sequence of LcRac1 shared more than 90% identity with other Rac1 counterparts (Fig. 2, Table 2). These results all suggested that Rac1 is highly conserved, and the LcRac1 protein is an ortholog of Rac1. The fact that LcRac1 contains a nuclear localization signal may indicate that it expressed at a relatively higher level in the nucleus. Additionally, fluorescence microscopy assays revealed that LcRac1 localized mainly in the nucleus fraction (Fig. 5).

As revealed in this study, the LcRac1 gene was constitutively expressed in the various tissues examined. This broad tissue expression pattern of LcRac1 is similar to the findings reported for human Rac1 [34], *Fc*Rac1 from shrimp (*Fenneropenaeus chinensis*) [21] and *Lv*Rac1 from shrimp (*Litopenaeus vannamei*) [22]. Notably, the most predominant expression of LcRac1 was found in heart, followed by blood, whereas the least was detected in the head-kidney (Fig. 6A and B). By comparison with other species, the corresponding ortholog were found

to be highly expressed in the gills and liver of turbot [35] and liver and heart of grass carp [36]. In Chinese shrimp, the highest expression was in hemocytes and moderate expression in the gills and intestine [21]. The differences in tissue distribution suggest that Rac1 implements a pivotal role in immune-related organs from teleosts to arthropods as hemocytes are mainly found in the lymphoid organ, one of the major immune organs in shrimp.

To clarify the role of LcRac1 in the immune responses of the large yellow croaker, we investigated the expression of Rac1 GTPase after infection of LPS, *V. parahaemolyticus* or poly I:C. This study showed that the LcRac1 gene expression was obviously up-regulated after stimulation with LPS, *V. parahaemolyticus* or poly I:C, indicating that it plays an important role in fish innate immunity against bacterial and viral infections. The spleen, liver, and head-kidney are the major sites of innate and adaptive immune responses in fish, and where a large number of immune cells including lymphocytes and macrophages are generated [37–39]. The present result was similar to the previous reports in Rac GTPase in large yellow croaker, where the gene was up-regulated in blood, spleen and liver after infection with LPS, poly I:C or *V. parahaemolyticus* [20]. Grass carp Rac1 mRNA was increased by *Aeromonas hydrophila* in several tissues [36].

It has been reported that the small GTPases might be involved in innate immunity. Phagocytosis plays a very important role in innate immunity and phagocytosis is a critical element of host defence against invading pathogens in higher organisms. Cytoskeleton proteins including β-actin and myosin are reported to be responsible for the phagocytosis of immune defences [20]. The interaction between LcRac1 and tropomyosin protein suggested that the LcRac1 protein might play an important role in phagocytosis [32]. Tropomyosin was sorted to different intracellular locations, often associated with actin filament populations that are involved in phagocytosis. To further clarify the role of LcRac1 in the phagocytosis response of *L. crocea*, we investigated the effects of LcRac1 silencing on the response to *V. alginolyticus* injection. LcRac1 expression was strongly suppressed in vivo by dsRNA-mediated gene silencing. In LcRac1-silenced fish challenged with *V. alginolyticus*, the expression of LcRac1 increased significantly at 48 hpi (Fig. 9A). As the phagocytosis assay showed that the phagocytic ability of hemocytes against to *V. parahemolyticus* was significantly affected by dsRNA-mediated LcRac1 silencing, LcRac1 knockdown greatly reduced bacterial clearance ability (Fig. 9B, C, D). Of note, Rac deletion in mice resulted in decreased B-cell maturation and T cell-independent antigen response [40,41]. And in the process of phagocytosis, Rac1 are recruited to the phagocytic site and converted from their inactive GDP-bound forms to their active GTP-bound forms, which is mediated by Fc receptors recognizing targets opsonized by immunoglobulin [42]. Rac1 is essential for the successful recruitment of the Arp2-Arp3 (Arp2/3) complex to the phagocytic cup [43]. In *Drosophila* larvae, Rac1 was involved in regulating hemocyte activation. Rac1 GTPase requires activation of Jun N-terminal kinase Basket (Bsk), as well as formation of stable actin to induce the *Drosophila* larvae cellular immune response [44]. In summary, these evidences suggested that LcRac1 can be induced by immune challenge and may play an important role in the innate immune response of the large yellow croaker to *V. parahemolyticus* infection.

In conclusion, a novel Rac1 GTPase was cloned and characterized from *L. crocea* for the first time. LcRac1 transcripts were ubiquitously expressed in multiple tissues with high levels in heart and blood. And the levels of mRNA of LcRac1 were up-regulated significantly after virus or bacteria stimulation. Additionally, tropomyosin protein was found to interact with LcRac1, indicating they functioned together to fight against pathogens infection. This discovery provided a better understanding of the roles of Rac1 in marine fishes.

Acknowledgements

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